FERTILIZATION AND EMBRYOGENY IN DIOON EDULE[1]

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(WITH PLATES XIV–XVII)

Fertilization in *Dioon edule* occurs from the second week in April to the second week in May. I have not been able to visit Mexico at this season of the year, but through the kindly influence of Gov. TEODORO A. DEHESA, who for several years has aided me in my investigation of Mexican cycads, and through the constant co-operation of Mr. ALEXANDER M. GAW, of the State Bureau of Information, Xalapa, Mexico, I have been able to secure material as regularly as if I had lived at Chavarrillo, where *Dioon edule* is most abundant. In cones picked at Chavarrillo, April 10, 1905, and examined at Chicago, April 21, nearly every nucellus contained one or more pollen tubes in which sperms were swimming vigorously; the next day there were not so many, and three days later nearly all pollen tubes had discharged their sperms; but as late as May 10 a few sperms were found. Consequently it would be possible for fertilization to occur at this late date, although not very probable, for all eggs capable of fertilization would probably have been fertilized already. A large cone picked at Chavarrillo, April 16, 1910, and examined at Chicago seven days later, contained embryos four times as long as the archegonia; consequently fertilization must have occurred as early as April 7. A few embryos in this cone were still in the free nuclear stage, but no pollen tubes were seen. These cones indicate the limits of the fertilization period as observed in the collections of six seasons. April 15 may be regarded as the optimum time, with fertilization frequent a week earlier and a week later, but becoming rare beyond these limits.

While fertilization may be somewhat delayed in cones sent to such a distance, the processes must be normal, for embryos

develop up to the cotyledon stage even in cones from which most of the sporophylls have been removed in securing material. In cones which arrive in Chicago with embryos in early cotyledon stages, the development is completed perfectly and the seeds will germinate.

Fertilization

The relations of the various structures at the time of fertilization are shown in fig. 1. The outer fleshy coat of the ovule has become highly differentiated, with its epidermal and hypodermal layers and deeper tissues containing tannin cells, mucilage ducts, and the outer vascular system. The stony coat has become so hard that it is difficult to cut it with a strong pocket knife; in fact, material of this and later stages was secured by sawing the ovule transversely with a fine saw. The inner fleshy coat has been reduced to a thin, papery membrane which, in the figure, appears as a dark border lining the inner surface of the stony layer. The nucellus, with its conspicuous beak and pollen tubes, has begun to sag. The tissue of the female gametophyte has become quite firm and contains numerous tannin cells, while the archegonial chamber has reached its maximum depth, and the nucleus of the central cell of the archegonium has just divided to form the ventral canal and egg nuclei. As stated in the paper on spermatogenesis in *Dioon edule* (8), the archegonial chamber is moist but contains no liquid before the pollen tubes begin to discharge. The sperms in escaping through the rather small rupture in the end of the pollen tube—there is no pit or pore—are much constricted, but soon regain their form and begin to swim. The amount of liquid discharged by a single pollen tube is small in comparison with the size of the archegonial chamber, and if the liquid should spread evenly, it would not be sufficient to cover the sperms; however, it behaves somewhat like a drop of water on a greasy surface, not spreading much, but moving until it comes into contact with the neck of an archegonium. The sperms move vigorously after being discharged from the pollen tube, but how long they might continue to swim under natural conditions in the archegonial chamber could not be determined. I had little success in keeping them alive in sugar solutions. When a sperm comes

into a sugar solution, the nucleus often slips out from the cytoplasmic sheath, and the cytoplasm then rinses off from the spiral band, leaving the band with its cilia almost free from any cytoplasm.

Before the pollen tubes have begun to discharge, the megaspore membrane over the archegonia has been ruptured, so that there is no obstruction between the sperms and the necks of the archegonia.

What causes the sperm to enter the egg? The classical experiments on chemotaxis would suggest a solution, but there is no free open passage as in bryophytes and pteridophytes, in which the small sperms may move freely in response to a slight stimulus. On the contrary the neck is closed, and the sperm becomes greatly constricted while entering, so that, whatever the force may be, it is a very strong one. Against the theory that chemotaxis is responsible for the movement, it must also be remembered that so skilful an investigator as MIYAKE (5) was unable to detect any chemotactic response when studying the sperms of *Cycas revoluta*, although he used the solutions which are uniformly effective with the sperms of pteridophytes, and in addition used the squeezed-out contents of the *Cycas* eggs.

It is well known that in many algae a small portion of the contents of the egg is extruded just before fertilization, a passage to the egg being facilitated in this way. In *Dioon*, just before fertilization, numerous preparations show a little protoplasm about the necks of the archegonia, and for a long time I assumed that it had been squeezed out from the egg by the pressure of the knife as the square block containing the archegonia was being cut out from the top of the endosperm before fixing. It was also noted that when material near the fertilization period is dropped into water or the fixing agent, a small bubble appears at the neck of the archegonium. The drop of liquid from the pollen tube has a very high osmotic pressure, and when it comes into contact with the extremely turgid neck cells, these lose so much of their contents that, in preparations, they appear more or less plasmolyzed. The pressure within the egg has been increasing until the contents are retained only by the rigidity of the turgid neck cells, and consequently even a slight decrease in turgidity of the neck cells would allow the escape of a small portion of the cytoplasm of the upper part of the egg, together,

probably, with some gas. In this way there is formed at the apex of the egg a vacuole, which may be of very short duration. In the absence of more definite knowledge, we suggest that this series of conditions would result in drawing the sperm into the egg, the cilia merely keeping it oriented, for sperms just within the egg showed the apex in advance in every case observed. It has already been noted in the paper on spermatogenesis (8) that the sperms have an amoeboid movement in addition to the movement by cilia. This amoeboid movement doubtless facilitates the entrance into the egg.

While the whole sperm enters the egg, the nucleus soon slips out from the cytoplasmic sheath and moves toward the egg nucleus, leaving the sheath with its ciliated band in the upper part of the egg. It may be that the constriction of the sperm during its entrance into the egg loosens the sheath from the nucleus so that it slips out more readily, for projections of various sizes extend from the nucleus into the sheath, and these would naturally become loosened. When more than one sperm enters the egg, it is worth noting that the nucleus of the second sperm does not slip out from the sheath, but remains intact in the cytoplasm at the top of the egg, where it may be distinguished to rather late stages in the development of the proembryo. Doubtless the first sperm opened the neck of the archegonium, so that the second suffered little constriction and the cytoplasmic sheath was not loosened. In some cases, at least, the sperm is still capable of vigorous movement after it is within the egg, as may be seen in fig. 1, where the contents of the egg have been stirred by the vibration of the cilia.

The cytoplasm of the sperm gradually mingles with that of the egg, and the two soon become indistinguishable, but the ciliated band maintains its identity much longer, and may be distinguished throughout the free nuclear development of the embryo, after which it becomes faint and finally merges with the cytoplasm of the upper part of the egg. As in *Zamia* (4), portions of the band occasionally penetrate farther, sometimes being found below the middle of the egg. The cytoplasm never surrounds the fusion nucleus, as in some gymnosperms, and there is no indication that it exerts any active influence upon the egg. That it does not stimulate is

evident from the fact that development is weakest in the upper portion of the egg.

The nucleus of the sperm enlarges somewhat during its passage to the egg nucleus, but details in the changes in its chromatin and changes in the chromatin of the egg nucleus were not studied. No preparation was secured which would show whether the chromatin contributions of the conjugating nuclei are distinct, as in *Pinus*, or whether the nuclei fuse in the resting condition, as is claimed for some gymnosperms. For a study of the nuclei at the time of fusion, *Ceratozamia* is a more favorable form, and consequently this feature will be investigated in that genus.

Embryogeny

HISTORICAL

The development of the cycad embryo has been studied in only two genera, *Cycas* and *Zamia*, and a few rather later stages have been noted in *Ceratozamia* and *Encephalartos*.

As early as 1877 WARMING (1) described some of the later stages in the development of the embryo of *Ceratozamia*, but in all cases the embryo had already passed through the base of the egg and invaded the endosperm. He noted the long suspensor and particularly the single cotyledon, a feature which Sister HELEN ANGELA (6) has since proved to be due to gravity, the missing cotyledon always appearing when developing seeds are rotated on a klinostat.

TREUB'S account of *Cycas circinalis* (2) in 1884 was comparatively full. A prolonged period of free nuclear division results in the formation of numerous free nuclei which are crowded to the periphery by a large central vacuole. Wall-formation then begins and the embryo, with its long suspensor, is organized from the cells at the base of the proembryo.

IKENO'S (3) account of *Cycas revoluta* confirms TREUB'S except in regard to the formation of the vacuole. IKENO finds that a large number of small vacuoles appear in the cytoplasm of the central portion of the egg, and that this central portion with its contained nuclei then disorganizes, leaving a parietal layer of protoplasm and nuclei, while a single large vacuole occupies the

center. After this stage, there are a few free nuclear divisions at the base of the egg before walls begin to appear, and at the same time amitotic divisions may take place in the upper portion.

COULTER and CHAMBERLAIN (4) found in *Zamia floridana* a somewhat different condition. After about eight simultaneous free nuclear divisions, giving rise to 256 free nuclei, walls appear at the base of the proembryo, just as in the formation of endosperm in most angiosperms, but no vacuole is developed.

SAXTON (9) has recently described some of the later stages in the embryo of *Encephalartos*. He finds that the suspensor is a root cap, and that the embryogeny is very similar to that of *Ginkgo*.

DIOON; FREE NUCLEAR PERIOD

In *Dioon edule* fertilization is followed immediately by a long period of simultaneous, free nuclear division. The first mitosis of the fusion nucleus occurs in the position where fertilization has taken place, a little above the middle of the egg, the spindle being oblique, as is usually the case in gymnosperms. The succeeding mitoses follow rapidly, and the nuclei, even as early as the 16-nucleate stage, become rather evenly distributed throughout the entire proembryo. Occasionally the nuclei move toward the bottom of the proembryo, the 16, 32, and 64-nucleate stages having been found with all the nuclei below the middle of the proembryo, and in one case a 64-nucleate stage had all the nuclei in the lower third. On the other hand, two 4-nucleate stages were observed with all the nuclei above the middle of the proembryo.

In stages following the 64-nucleate stage—resulting from the sixth mitosis—the number of nuclei in the lower part of the proembryo is likely to be considerably larger than that in the upper part. At the 128 and 256-nucleate stages—resulting from the seventh and eighth mitoses—the larger number at the bottom is due to a settling of the nuclei; but at the ninth mitosis some of the nuclei in the upper half of the proembryo may fail to divide, and thus add to the disproportion. It may happen, occasionally, that the ninth mitosis, which gives rise to the 512-nucleate stage, may be the last of the simultaneous divisions; but usually there is a tenth mitosis, which would give rise to 1024 nuclei, if all nuclei

should divide at every simultaneous mitosis. A rough estimate, based upon fig. 6, shows that the evanescent walls, to be described later, are those resulting from the tenth mitosis; and fig. 7, in which permanent walls have been established, shows that in this proembryo the permanent walls appeared in connection with the tenth mitosis. We have not found any case which would indicate an eleventh mitosis, and only a few cases where permanent walls seem to have been formed at the ninth mitosis; while nearly all proembryos in approximately the stage shown in fig. 7 indicate that there have been ten mitoses with permanent walls beginning in connection with the tenth.

During the period of free nuclear division, the figures are entirely intranuclear and are characterized by the abundance and persistence of the spindle fibers (figs. 2–4). The nuclear membrane is formed quite late (fig. 3), but when once formed it becomes unusually thick, and pieces of it may be seen until late prophase or early metaphase of the next mitosis (fig. 2, *n*).

DIOON; EVANESCENT SEGMENTATION

The most characteristic feature of the proembryo of *Dioon edule* is the complete but evanescent segmentation of the entire egg. In one case, as early as the 64-nucleate stage, faint walls were observed dividing the entire proembryo into uninucleate cells. Such walls break down completely before the next mitosis begins. At the seventh and eighth mitoses, giving rise to the 128 and 256-nucleate stages, the appearance of walls is more frequent and they break down less rapidly; while at the ninth mitosis, giving rise to the 512-nucleate stage, the walls are probably always formed; and at the tenth division the walls are formed; but while they disappear in the upper portion of the proembryo, leaving it in free nuclear condition, they persist at the base, constituting the first permanent walls. As soon as the proembryo becomes cellular, the simultaneous mitoses are succeeded by the scattered mitoses which one expects in a young cellular embryo.

The evanescent walls consist of broad bands of spindle fibers, about two-fifths of the spindle nearest the daughter nuclei disappearing very early, while the middle fifth remains long enough to

cause a segmentation which is so conspicuous that it is seen easily with a pocket lens (figs. 5, 6).

The evanescent walls were, for a time, the most puzzling feature in the embryogeny, especially while it was thought that they belonged exclusively to some particular mitosis. As observations multiplied and it became evident that the evanescent segmentation might occur at the sixth mitosis and was increasingly frequent at the seventh, eighth, and ninth mitoses, the explanation became evident. The early mitoses, up to the sixth, follow in such rapid succession that a new mitosis is begun before the preceding one could establish a wall. Between the succeeding mitoses the intervals are increasingly greater, and consequently the natural tendency of a spindle to develop a wall finds a more marked expression.

We regard the appearance of evanescent walls as a reversion, indicating that the ancestors of *Dioon* had embryos which were cellular throughout. It can hardly be questioned that the free nuclear condition in gymnosperms, whether in the proembryo or in the gametophyte, is not a primitive but rather a highly specialized condition. In the small eggs of the pteridophytes, including even the heterosporous genera, the first nuclear division is followed by the formation of a wall. We suggest that free nuclear division in the proembryo of gymnosperms arose in connection with the increasing size of the egg, the increased mass of the egg becoming too large to be segmented, and at the same time the large mass favoring rapidly succeeding mitoses. The nuclei divide simultaneously because exposed to practically similar conditions. As mitosis continues and the mass of cytoplasm about each nucleus becomes less, the mitoses succeed each other less rapidly, and as the relation between the nuclei and surrounding cytoplasm approaches that which is found in the ordinary tissues, segmentation begins. If this view is correct, we should expect the most extensive free nuclear periods in the largest eggs, and such is certainly the case. The egg of *Dioon edule* often reaches a length of 5 mm., and the number of free nuclei is about 1000, theoretically 1024. *Zamia*, with an egg about 3 mm. in length, has 256 free nuclei; and *Gingko*, with an egg of somewhat approximately the

same mass, shows the same number. The much smaller eggs of Coniferales have comparatively short free nuclear periods, showing only 32, 16, 8, 4, and 2 free nuclei; while in the small egg of *Sequoia* the first mitosis is followed by a wall.

While it might be tempting to regard this as a reduction series, and to regard forms with a large number of free nuclei as primitive, it seems probable that the number of free nuclei is correlated merely with the size of the egg. That the large number does not prove antiquity is shown by the fact that *Dioon* has 1000 free nuclei, while *Pinus* has only 8 (i.e., walls appear in connection with the 8-nucleate stage), and yet *Pinus* is much more ancient than *Dioon*. We should expect to find the critical forms among the lower Cycadofilicales as they began to be differentiated from some heterosporous fern ancestry. These forms, which are still to be discovered, should have small eggs with complete segmentation not preceded by any free nuclear period, the free nuclear feature appearing later in connection with an increased size of the egg. Although this is highly theoretical, we believe that it suggests an explanation of the structures as we find them in living gymnosperms.

DIOON; THE CELLULAR EMBRYO

At the last mitosis of the free nuclear or evanescent segmentation period, permanent walls are developed at the base of the proembryo, the walls in the upper part being evanescent and disappearing as before. The nuclei in this upper region do not divide any more and many of them disorganize. In the basal region nuclear division, always accompanied by cell division, progresses rapidly; and while one can still estimate with some degree of exactness the extent to which the free nuclear stage has progressed, a small-celled tissue is formed (fig. 7). At the stage shown in this figure, there is practically no indication of regions corresponding to the "rosette," suspensor, and embryo proper regions of many conifers. At this stage the archegonium jacket is still conspicuous, and at the base of the proembryo the jacket has become extended into a tissue several cells deep, contrasting sharply with the adjacent starchy cells of the endosperm. Cellulose walls have formed on the peripheral surfaces of the cells of this proembryo and the egg membrane is still intact.

Very soon after the stage shown in fig. 7, a differentiation becomes apparent. A thick wall forms a sort of diaphragm between the definitely cellular and the free nuclear or loosely cellular portions of the proembryo (figs. 8, 9, *d*). Just below the diaphragm the cells are large and with scanty protoplasmic contents, while one and in the middle two layers of cells are much smaller and have dense protoplasmic contents (fig. 8). This more deeply staining region at the tip becomes the embryo proper, while the large cells, in contact with the diaphragm, will differentiate into the suspensor. Long cells like *a* and *b* in fig. 8 may divide again, the outer cell then contributing to the suspensor, while the other forms a part of the "rosette" and never gets beyond the original limits of the egg. The suspensor cells now divide both longitudinally and transversely, and it becomes difficult or impossible to distinguish any boundary between suspensor and embryo proper (fig. 9). As the periclines at the tip of this figure indicate, the dermatogen has not yet been differentiated. In both figs. 8 and 9 the embryos have not yet pierced the egg membrane.

The egg membrane now disorganizes, apparently becoming digested, and the rapidly elongating suspensor thrusts the embryo through the base of the egg into the endosperm. At this time mucilage ducts begin to appear in the suspensor (fig. 10). As soon as the embryos break through the egg membranes, the four or five or six suspensors become twisted together, and as one dissects them out they appear like a single suspensor with one embryo at the tip; but when they have reached a length of 1 cm., a careful examination with a pocket lens will show unsuccessful embryos at various distances from the egg region and a single embryo at the tip. I have never seen a suspensor branching, as described for *Encephalartos* by SAXTON (9), but have seen unsuccessful embryos which project somewhat from the general suspensor and so caused the appearance of branching.

The suspensor is a remarkable feature of the cycad embryo. Its growth and twisting is accompanied by an extensive disorganization of the endosperm in the archegonial region, especially just beneath the archegonia, so that there is formed a large cavity which becomes filled with the long suspensor. The cavity is moist,

but not full of liquid. The cells disorganized in the formation of the cavity must have contributed largely to the growth of the suspensor, since the embryo itself is receiving from surrounding cells enough to account for any increase in its size. That the suspensor exerts a vigorous thrust is evident from the fact that when released it relaxes like a spring which has been under tension. The suspensor when stretched out reaches a length of 75 mm. As the embryo matures, the suspensor does not disorganize, but remains as a tough dry thread, strong enough to lift the weight of the entire embryo.

The embryo develops rapidly, cell division taking place throughout the entire embryo, while the suspensor cells elongate and also occasionally undergo mitosis (figs. 10–12). In the last figure the two upper mitoses are in the suspensor and the three lower in the embryo proper. It is interesting to note that such mitoses are found in cones which have been taken from the plant in southern Mexico, shipped to Chicago in a basket, and after arriving have been lying on the laboratory table for two or three weeks.

The first indication of any differentiation of the embryo proper is the appearance of increased vigor in cell division in the region just below the suspensor, indicated by x in fig. 14. This region, which appears in the embryo and not in the suspensor, is the beginning of the coleorhiza, an organ which rapidly becomes conspicuous and soon constitutes the largest part of the embryo (figs. 15, 16).

While the coleorhiza and the root cap are spoken of as two organs, and while they differ widely in general appearance, in cell structure and in function they are morphologically only one organ, the root cap. The coleorhiza at first consists of thin-walled cells with numerous large starch grains, but toward the close of the intraseminal period the cell walls become very hard and thick, so that the term coleorhiza is quite appropriate. Just within the coleorhiza the mature embryo shows a cone of thin-walled cells, rich in tannin, derived from the same meristem, the beginning of which is seen in fig. 14. These cells are the outer layers of the root cap. At germination the pressure is developed, at least in large part, by the activity of the meristem which is adding new

layers to the root cap. The pressure, crowding the coleorhiza against the micropylar end of the seed, causes an irregular jagged fracture of the stony coat of the seed, through which the embryo emerges. It is only after the fracture is large enough to permit the exit of the embryo that the root cap pierces the central portion of the coleorhiza, the thick-walled cells of which may be seen clinging to the root in threadlike masses even after the axis of the seedling has become vertical.

The late differentiation of body regions is a noticeable feature of the cycad embryo. At the stage shown in fig. 15, not only is the differentiation into periblem and plerome still incomplete, but even the dermatogen is not yet differentiated, as may be seen by the periclines in the cotyledons and especially in the stem tip (figs. 15, 17). Perhaps the importance of division by anticlines only has been overestimated, for in this embryo the outer layer looks like a dermatogen long before the periclines cease to appear.

A general view of an embryo at the stage shown in fig. 15 may be seen in the accompanying diagram (fig. 16), drawn from the same preparation. Just below the suspensor with its large mucilage cavities is the swollen coleorhiza. Starch is found in the suspensor, but is most abundant in the peripheral and lower regions of the coleorhiza. Cell division is most active in the dotted region of the stem tip. While the general appearance of the mitoses is very different from that described for the free nuclear period, it is probable that the difference is not so great as it seems, for in fig. 18 the spindle may mark the limit of the nuclear membrane.

A diagrammatic section through a ripe seed is shown in fig. 19. The embryo extends almost the entire length of the seed, the cotyledons constituting the principal mass. The first functional leaf, if drawn on a large scale, would show the primordia of pinnules, the scale leaves not showing such a differentiation. The outer fleshy layer and the middle or stony layer (shaded) are easily distinguished, but the inner fleshy layer, while forming a conspicuous membrane containing the inner vascular system, is too thin to be shown in the diagram. Although the stony layer is quite thin in the region of the pit at the base of the seed, the cotyledons never emerge at

that point, but remain in the seed, sometimes until the seedling is three or four years old.

The embryo may develop continuously, germinating without any resting period, or may germinate after a resting period. Seeds which had been in the laboratory for two years germinated readily, but how long they might retain their power of germination is uncertain.

While the general course of development in the later stages of the embryo is the same in all of my material (*Dioon*, *Ceratozamia*, *Zamia*, *Microcycas*, and *Encephalartos*), there are considerable differences in details which may prove suggestive, and consequently no more detailed description will be given until a comparative study has been made.

The development of the vascular system of the embryo and seedling of *Dioon edule* has been investigated in this laboratory by THIESSEN (7).

Summary

1. The liquid from the pollen tube causes a decrease in the turgidity of the neck cells, and this allows a portion of the upper part of the egg to escape, thus forming a vacuole at the top of the egg. The entire sperm is thus drawn into the egg.

2. There are sometimes nine, but oftener ten simultaneous free nuclear divisions, resulting in the formation of 512 or 1024 free nuclei.

3. There is a complete but evanescent segmentation of the entire proembryo, the walls then disappearing except at the base of the proembryo, where they become permanent.

4. The coleorhiza and root cap, although differing in appearance, cell structure, and in function, are formed from the same meristem, and both are morphologically root cap.

5. Plerome, periblem, and dermatogen are differentiated quite late in the development of the embryo.

6. The seed germinates without any resting period, but also has been germinated after a rest of two years.

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DESCRIPTION OF PLATES XIV–XVII

FIG. 1.—Upper part of ovule at the time of fertilization; the figure is reconstructed from several sections; the pollen tube on the left shows the body cell still undivided; the one in the middle shows two sperms and the remains of the stalk and prothallial cells; next to this tube is one in which the body cell has just divided, and the prothallial and stalk cells are still turgid; two pollen tubes which have discharged their contents are also shown; the dark line below the outer part of the nucellus is the megaspore membrane.

FIG. 2.—Late prophase during the free nuclear division in the proembryo, showing the intranuclear membrane (*n*). ×480.

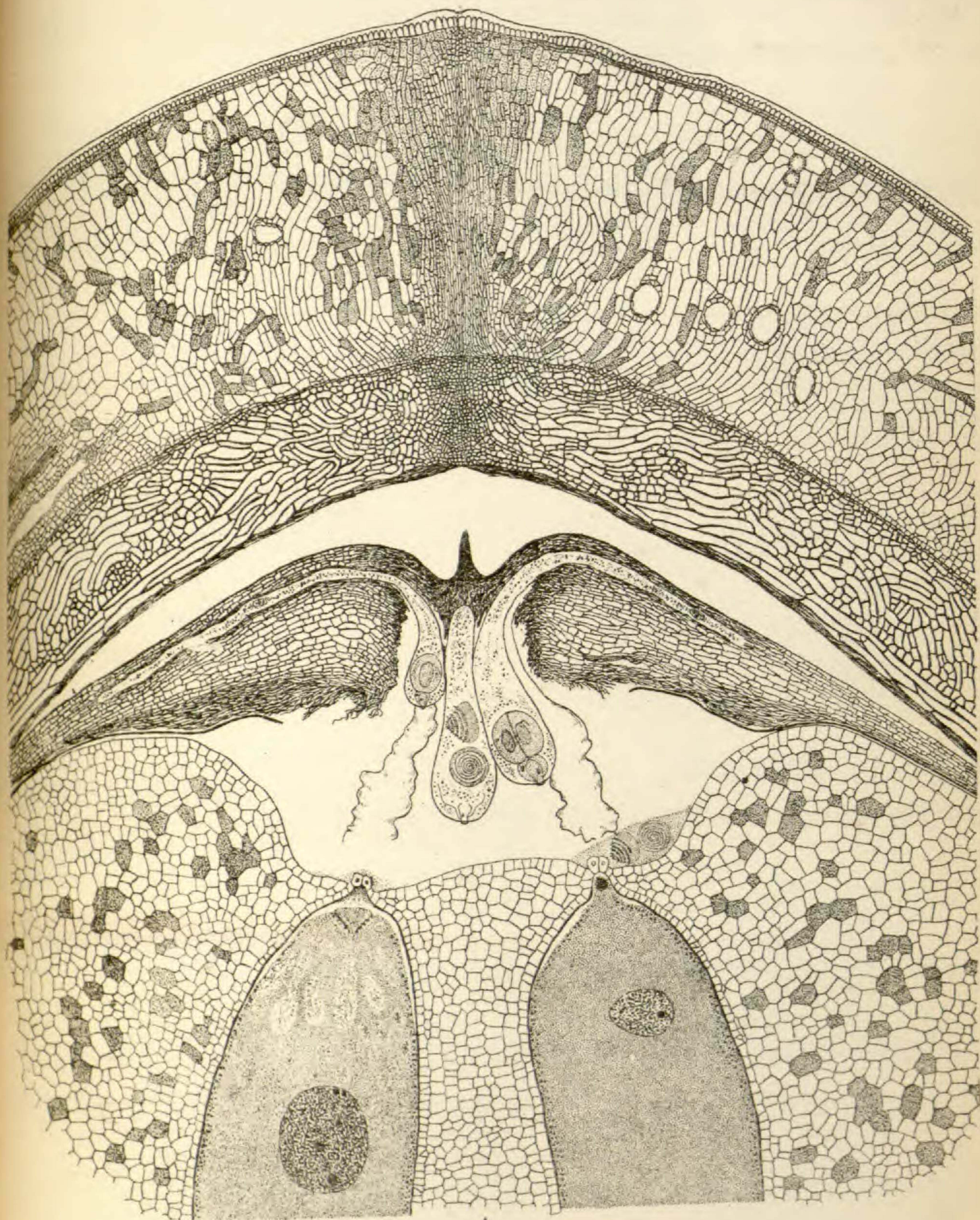FIG. 3.—Early telophase during the free nuclear period; the nuclear membrane has not yet been formed. ×480.

FIG. 4.—Telophase during free nuclear period; the central portion of the fibers from the evanescent walls. ×480.

FIG. 5.—Lower part of proembryo, showing evanescent but complete segmentation.

FIG. 6.—Diagram showing the complete proembryo of which fig. 5 shows only the lower part.

FIG. 7.—Lower part of proembryo, showing permanent walls at the base.

FIG. 8.—Young embryo: *d*, diaphragm; *f*, free nuclei; *a* and *b*, cells which may contribute to both "rosette" and suspensor.

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